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December 14, 2001

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Commissioner for Patents
Washington, D.C. 20231

PCT/JP00/03896
-filed June 15, 2000

Re: Application of Shingo KATO, Yoshio KOBAYASHI, Yoshiyuki HIRAISHI, Kayoko SHIMIZU and Tetsuyoshi SUGITA
METHOD FOR DETERMINING HIV-1 SUBTYPE
Assignee: OTSUKA PHARMACEUTICAL CO., LTD. and KEIO UNIVERSITY
Our Ref: Q67685

Dear Sir:

The following documents and fees are submitted herewith in connection with the above application for the purpose of entering the National stage under 35 U.S.C. § 371 and in accordance with Chapter II of the Patent Cooperation Treaty:

- ☑ an executed Declaration and Power of Attorney.
- ☑ an English translation of the International Application.
- ☑ ten (10) sheets of drawings.
- ☑ twelve (12) pages of Sequence Listing and a 3.5" disk containing Sequence Listing.
- ☑ Statement in Support of Submission in Accordance with 37 C.F.R. § 1.821.
- ☑ an executed Assignment and PTO 1595 form.
- ☑ Information Disclosure Statement and a Form PTO-1449.

It is assumed that copies of the International Application, the International Search Report, the International Preliminary Examination Report, and any Articles 19 and 34 amendments as required by § 371(c) will be supplied directly by the International Bureau, but if further copies are needed, the undersigned can easily provide them upon request.

The Government filing fee is calculated as follows:

Total claims	18	-	20	=		x $18.00	= $.00
Independent claims	2	-	3	=		x $84.00	= $.00
Base Fee							$890.00
TOTAL FILING FEE							$890.00
Recordation of Assignment							$ 40.00
TOTAL FEE							$930.00

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Checks for the statutory filing fee of $890.00 and Assignment recordation fee of $40.00 are attached. You are also directed and authorized to charge or credit any difference or overpayment to Deposit Account No. 19-4880. The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16, 1.17 and 1.492 which may be required during the entire pendency of the application to Deposit Account No. 19-4880. A duplicate copy of this transmittal letter is attached.

Priority is claimed from:

Country	Application No	Filing Date
Japan	1999-167736	June 15, 1999
Japan	2000-23581	February 1, 2000

Respectfully submitted,

Gordon Kit
Registration No. 30,764

GK/amt

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Shingo KATO et al

CHAPTER II of
Appln. No.: **PCT/JP00/03896** Group Art Unit: **0000**

Filed: **December 14, 2001** Examiner: **Unknown**

For: **METHOD FOR DETERMINING HIV-1 SUBTYPE**

STATEMENT IN SUPPORT OF SUBMISSION
IN ACCORDANCE WITH 37 C.F.R. § 1.821

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

The following statement is provided to meet the requirements of 37 C.F.R. § 1.821.

I hereby state that the content of the computer readable copy (PatentIn Version 2.1) of the Sequence Listing (attached hereto) submitted in accordance with 37 C.F.R. §§ 1.821(c) and (e), respectively, is the same as the Sequence Listing submitted herewith.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge and that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

12/14/01
Date

Gordon Kit

DESCRIPTION

Method for Determining HIV-1 Subtype

TECHNICAL FIELD

The present invention relates to a method for determining HIV-1 subtypes, and a kit for determining HIV-1 subtypes.

BACKGROUND ART

The virus causing acquired immune deficiency syndrome (AIDS) is the human immunodeficiency virus (HIV), of which type 1 (HIV-1) and type 2 (HIV-2) are known. Most cases involve HIV-1, for which various subtypes have been discovered.

Determining the HIV-1 subtype in infected individuals is important for assessing the route of infection and the reliability of virological test results (particularly the drug resistance based on genotype or the determination of plasma HIV-1 RNA concentration). HIV-1 subtypes are generally determined through the sequencing of specific regions of the virus genome and phylogenetic analysis of the results, but these are complicated and expensive procedures.

An object of the present invention is thus to provide a simpler method for determining HIV-1 subtypes.

Another object of the present invention is to provide a kit for determining HIV-1 subtypes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates nucleotide sequences of the 5' adjacent region (C2 region) of the V3 region for the env gene in various subtypes of HIV-1. Capital letters indicate nucleotides that are entirely the same within a given subtype, and lower case letters indicate nucleotide variants within a given subtype. A question mark indicates that a consensus nucleotide was not determined because of too many variants. A dash indicates a nucleotide identical to that in subtype A. A period indicates the absence of a nucleotide in the corresponding site.

Figure 2 illustrates nucleotide sequences of the 3' adjacent region (C3 region) of the V3 region for the env gene in various subtypes of HIV-1. Capital letters indicate nucleotides that are entirely the same within a given subtype, and lower case letters indicate nucleotide variants within a given subtype. A question mark indicates that a consensus nucleotide was not determined because of too many variants. A dash indicates a nucleotide identical to that in subtype A. A period indicates the absence of a nucleotide in the corresponding site.

Figure 3 illustrates the locations, combinations, and base sequences of primers used in nested PCR

(different primer pairs used for first and second PCR) for determining HIV-1 subtypes.

Figure 4 gives the results obtained when subtypes were detected by nested PCR using the primers illustrated in Figure 3 for samples in which the subtypes had been determined by sequencing of the virus genome.

Figure 5: Location of primers in HIV-1 subtype-specific nested PCR

Figure 5 illustrates the locations, combinations, and base sequences of primers used in nested PCR for determining HIV-1 subtypes. 9M indicates a mixture of primers 9AE and 9B; 11M indicates a mixture of primers 11LAE, 11LB, and 11LC; and 12M indicates a mixture of primers 12A and 12B.

Figure 6: Subtype-specific PCR of HIV-1 DNA.

Figure 6 gives the results obtained when subtypes were detected by nested PCR using the primers illustrated in Figure 5.

Figure 7: Phylogenetic analysis of HIV-1 variants

Figure 7 gives the results of subtypes obtained by phylogenetic analysis of HIV-1 variants based on the base sequence of the V3 region of the env gene obtained through sequencing.

Figure 8: Amino acid sequence in PR of non-subtype B HIV-1 in patients receiving HAART

Figure 8 illustrates the amino acid sequences related to protease inhibitor resistance in non-subtype B HIV-1 patients receiving HAART.

Figure 9 is a table of the correlation between various subtypes and the sexual behavior of HIV-1 patients.

Figure 10: RT-PCR of RNA from PA^+ but WB^- plasma with universal primers

Figure 10 gives the results obtained in RT-PCR using primer pairs allowing HIV-1 to be amplified irrespective of subtype in samples of serum from patients diagnosed as particle adsorption-positive (PA^+) but Western blotting negative (WB^-). N1 and N2 are negative controls, while P1 and P2 are positive controls.

DISCLOSURE OF THE INVENTION

The inventors have designed various subtype-specific primers and have successfully used them to amplify nucleic acid in samples for rapid determination of HIV-1 subtypes, thereby perfecting the present invention.

Specifically, the present invention provides a method for determining HIV-1 subtypes, characterized by comprising the steps of amplifying nucleic acid using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1, where at least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the HIV-1 subtype, and detecting the subtype

depending on whether or not the nucleic acid has been amplified. The target sequence should be 100 to 2500 base pairs in length, and preferably 150 to 500 base pairs in length. In the above method, the sequence from the 1st through 30th bases from the 3' terminal and/or 5' terminal of the target sequence should be different depending on the subtype. For example, the 3' terminal of the target sequence may be the C3 region of the env gene of HIV-1. The 5' terminal of the target sequence may be the C2 region of the env gene of HIV-1. Different subtypes can be detected by different amplification reactions using different primer pairs. For example, at least two subtypes can be distinguished following amplification carried out at least twice with two different pairs of primers of which 3' primers (primers 1) include sequences complementary to portions of the nucleotide sequence (nucleotide sequence 1) that differs depending on subtype in the C3 region of the env gene of HIV-1, and 5' primers (primers 2) include sequences complementary to portions of the nucleotide sequence (nucleotide sequence 2) of the C2 region of the env gene of HIV-1.

A first amplification reaction may be carried out with a first pair of primers using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1, a second amplification reaction may then be carried out

with a second pair of primers using as a target sequence a portion of the aforementioned nucleotide sequence, where at least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the HIV-1 subtype, and the subtype may be detected depending on whether or not the nucleic acid has been amplified by the second amplification reaction. For example, the second pair of primers may consist of a primer (primer 1) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 1) that differs depending on subtype in the C3 region of the env gene of HIV-1, and a primer (primer 2) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 2) of the C2 region of the env gene of HIV-1; and the first pair of primers may consist of a primer (primer 3) that includes a sequence complementary to a portion of a nucleotide sequence (nucleotide sequence 3) of a region downstream of the 3' terminal of nucleotide sequence 1 of the env gene of HIV-1, and a primer (primer 4) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 4) of a region upstream of the 5' terminal of nucleotide sequence 2 of the env gene of HIV-1.

At least two subtypes can also be distinguished by repeating at least once the following series of operations

with different pairs of second primers, where the operations comprise a first amplification reaction that is carried out with the first pair of primers using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1, a second amplification reaction that is then carried out with the second pair of primers using as a target sequence a nucleotide sequence within the above target sequence, and the detection of subtypes depending on whether or not the nucleic acid has been amplified by the second amplification reaction. For example, subtypes A, B, C, and E can be distinguished by: (a) detecting subtype A using as the first primer pair a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6), and a mixture of primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair primer 11QA1 containing nucleotide sequence CTCCTGAGGAGTTAGCAAAG (Sequence ID No. 27) and primer 10U containing nucleotide sequence CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20);

(b) detecting subtype B using as the first primer pair a mixture of primer 12A containing nucleotide

sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6), and a mixture of primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair primer 11VB containing nucleotide sequence CACAATTAAAACTGTGCATTAC (Sequence ID No. 28) and primer 10U containing nucleotide sequence CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20);

(c) detecting subtype C using as the first primer pair a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6), and a mixture of primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair primer 11XC containing nucleotide sequence TTGTTTTATTAGGGAAGTGTTC (Sequence ID No. 29) and primer 10U containing nucleotide sequence CTGTTAAATGGTAGTCTAGC (Sequence ID No. 24); and

(d) detecting subtype C using as the first primer

pair a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6), and a mixture of primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair primer 11WE containing nucleotide sequence CTCTACAATTAAAATGATGCATTG (Sequence ID No. 30) and primer 10U containing nucleotide sequence CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20).

Alternatively, at least two subtypes can be distinguished by repeating at least once the following series of operations with different pairs of first and second primers, where the operations comprise a first amplification reaction that is carried out with a first pair of primers using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1, a second amplification reaction that is then carried out with a second pair of primers using as a target sequence a nucleotide sequence within the target sequence in the first reaction, and the detection of subtypes depending on whether or not the nucleic acid has been amplified by the second amplification reaction. For example, subtypes A, B,

and E can be distinguished by: (a) detecting subtype A using as the first primer pair primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8), and using as the second primer pair primer 11QA containing nucleotide sequence CTCCTGAGGGGTTAGCAAAG (Sequence ID No. 1) and primer 10 containing nucleotide sequence AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4);

(b) detecting subtype B using as the first primer pair primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair primer 11BB containing nucleotide sequence CTGTGCATTACAATTTCTGG (Sequence ID No. 2) and primer 10 containing nucleotide sequence AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4); and

(c) detecting subtype E using as the first primer pair primer 12E containing nucleotide sequence GCAATAGAAAAATTCCCCTC (Sequence ID No. 7) and primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8), and using as the second primer pair primer 11QE containing nucleotide sequence CTCCTGAGGGTGGTTGAAAG (Sequence ID No. 3) and primer 10

containing nucleotide sequence AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4).

The method of the present invention may further comprise the steps of amplifying nucleic acid using as a target sequence a portion of a nucleotide sequence of the HIV-1 genome, the nucleotide sequence being highly conserved among all subtypes, and ascertaining the presence or absence of HIV-1 depending on whether or not the nucleic acid has been amplified. The step for ascertaining the presence or absence of HIV-1 comprises amplifying the nucleic acid with a first primer pair using as a target sequence a portion of a nucleotide sequence of the HIV-1 genome, the nucleotide sequence being highly conserved among all subtypes, then carrying out a second amplifying reaction with a second primer pair using as a target sequence a nucleotide sequence in the above target sequence, and ascertaining the presence or absence of HIV-1 depending on whether or not the nucleic acid has been amplified. The first primers referred to here may comprise a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5), primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6), primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8), and primer 9B nucleotide sequence CACAGTACAATGTACACATG

(Sequence ID No. 9), and the second primer pair may comprise primer 11LB containing nucleotide sequence AATTTCTGGGTCCCCTCCTG (Sequence ID No. 18), primer 11LAE containing nucleotide sequence AATTTCTAGATCCCCTCCTG (Sequence ID No. 25), primer 11LC containing nucleotide sequence AATTTCTAGGTCCCCTCCTG (Sequence ID No. 26), and primer 10U containing nucleotide sequence CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20).

Another object of the present invention is to provide a kit for determining HIV-1 subtypes, comprising primer pairs in which a target sequence is a portion of a nucleotide sequence of the env gene of HIV-1, where at least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the subtype.

Embodiments of the present invention are illustrated below.

A sample of blood, lymph, spinal fluid, semen, lymph node, or the like is taken from individuals suspected of HIV-1 infection, infected individuals and patients confirmed with HIV-1 infection, patients being treated for HIV-1, and the like. DNA is extracted using a QIAamp Blood Kit by QIAGEN, either directly or after monocytes have been isolated from the sample by Ficoll-Paque gradient centrifugation (Pharmacia). Alternatively, RNA is extracted using a QIAamp Viral RNA Kit by QIAGEN from

plasma. The DNA or RNA concentration is then determined base on the optical resolution at 260 nm.

The nucleic acid is then treated in PCR, and preferably nested PCR.

The use of nested PCR is described below. Nested PCR involves designing a second primer pair inside a target sequence amplified with another primer pair (first primer pair), carrying out a first PCR step, and then carrying out a second PCR step, and then diluting the reaction product as new template for a second PCR step. Undesirable sequences are sometimes amplified in addition to the target sequence in the first PCR step. However, there is very little probability that undesirable fragments amplified during the first PCR step will have a sequence with which the primers of the second primer pair will anneal. The second PCR step is thus carried out for selective amplification of only the target sequence.

The initial PCR step (first PCR) is first carried out using different primers for each subtype to be distinguished (such as subtype A, subtype B, and subtype E). Alternatively, universal primer pairs allowing any type of subtype to be amplified can be used instead of subtype-specific primer pairs.

An example of a subtype-specific primer pair is a primer pair consisting of a primer (primer 4') which

includes a sequence complementary to a portion of a nucleotide sequence in the C2 region of the env gene of HIV-1 and a primer (primer 3') which includes a sequence complementary to a portion of the nucleotide sequence of the C3 region of the env gene for HIV-1 that differs depending on subtype (that is, subtype-specific nucleotide sequence). Since the C2 region of the env gene of HIV-1 has a nucleotide sequence that differs depending on the subtype, as shown in Figure 1, the nucleotide sequence may be selected from this region to design primer 4'. Because the C3 region of the env gene of HIV-1 varies depending on the subtype, as shown in Figure 2, the nucleotide sequence may be selected from this region to design primer 3'. The primer length should generally be 18 to 30 base pairs, and preferably 20 to 25 base pairs. The following primers can be specifically used.

Primer pairs and their nucleotide sequences for first PCR to detect subtype A

9AE/12A

primer 9AE: CACAGTACAATGCACACATG (Sequence ID No. 8)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

Primer 9AE is a subtype A, E, F, and H-specific primer in which the sequence is complementary to the sequence from 6943 to 6962, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1

(NL4-3 strain).

Primer 12A is a subtype A, C, E, G, H, I, and J-specific primer in which the sequence is complementary to the sequence from 7369 to 7350, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer pairs and their nucleotide sequences for first PCR to detect subtype B

9B/12B

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12B: ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6)

Primer 9B is a subtype B, C, D, E, F, G, H, and J-specific primer in which the sequence is complementary to the sequence from 6943 to 6962, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 12B is a subtype B, D, E, F, and I-specific primer in which the sequence is complementary to the sequence from 7369 to 7350, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer pairs and their nucleotide sequences for first PCR to detect subtype E

9AE/12E

primer 9AE: CACAGTACAATGCACACATG (Sequence ID No. 8)

primer 12E: GCAATAGAAAAATTCCCCTC (Sequence ID No. 7)

Primer 12E is a primer specific to subtype E only, in which the sequence is complementary to the sequence from 6943 to 6962, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer pairs and their nucleotide sequences for first PCR to detect subtype C

9B/12A

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

Primer pairs and their nucleotide sequences for first PCR to detect subtype D

9B/12B

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12B: ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6)

Primer pairs and their nucleotide sequences for first PCR to detect subtype F

9B/12A

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

Primer pairs and their nucleotide sequences for first PCR to detect subtype G

9B/12A

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

Primer pairs and their nucleotide sequences for first PCR to detect subtype H

9B/12A

primer 9B: CACAGTACAATGTACACATG (Sequence ID No. 9)

primer 12A: GCAATAGAAAAATTCTCCTC (Sequence ID No. 5)

First PCR may alternatively be carried out using a primer mixture capable of giving amplified products for several subtypes. An example of a primer for such a purpose is a mixture of primers 9AE, primer 9B, primer 12A, and primer 12B.

1/1000 to 1/5 (for example, 1/50) of the PCR products is used to carry out the next PCR (second PCR) with another pair of primers that differ by subtype. The pair of primers for second PCR is designed from within the target sequence amplified during the first PCR. For example, at least one of the primers forming the subtype-specific primer pair for second PCR can be a primer (primer 1) containing a sequence complementary to a portion of the subtype-specific nucleotide sequence of the C2 region of the env gene for HIV-1. Because the nucleotide sequence of the C2 region of the env gene for HIV-1 differs by subtype, as shown in Figure 1, a nucleotide sequence from this region can be selected to design the primer. Figure 2 gives the nucleotide sequence

of the 3' adjacent region (C3 region) of the V3 region of the env gene for various subtypes of HIV-1. Since the nucleotide sequence varies depending on the subtype, a suitable sequence can be selected to design a primer. To design a subtype-specific primer, phylogenetic analysis is employed to select nucleotide sequences of a given subtype which are as genetically remote as possible from the corresponding nucleotide sequence of other subtypes. An example can include a primer (primer 2) containing a sequence complementary to a portion of the nucleotide sequence of the C3 region of the env gene for HIV-1. The primers pairs containing the following nucleotide sequences are specific examples.

Primer pairs and their nucleotide sequences for second PCR to detect subtype A

10/11QA

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11QA: CTCCTGAGGGGTTAGCAAAG (Sequence ID No. 1)

Primer 10 is a subtype A, B, D, and E-specific primer in which the sequence is complementary to the sequence from 6997 to 7016, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11QA is a primer specific to only subtype A,

in which the sequence is complementary to the sequence from 7313 to 7294, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

10U/11QA1

primer 10U: CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20)

primer 11QA1: CTCCTGAGGAGTTAGCAAAG (Sequence ID No. 27)

Primer 10U is a subtype A, B, D, E, and J-specific primer in which the sequence is complementary to the sequence from 6992 to 7011, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11QA1 is a primer specific to only subtype A, in which the sequence is complementary to the sequence from 7313 to 7294, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer pairs and their nucleotide sequences for second PCR to detect subtype B

10/11BB

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11BB: CTGTGCATTACAATTTCTGG (Sequence ID No. 2)

Primer 11BB is a primer specific to only subtype B, in which the sequence is complementary to the sequence

from 7338 to 7319, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

10U/11VB

primer 10U: CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20)

primer 11VB: CACAATTAAAACTGTGCATTAC (Sequence ID No. 28)

Primer 11VB is a primer specific to only subtype B, in which the sequence is complementary to the sequence from 7349 to 7328, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer pairs and their nucleotide sequences for second PCR to detect subtype E

10/11QE

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11QE: CTCCTGAGGGTGGTTGAAAG (Sequence ID No. 3)

Primer 11QE is a primer specific to only subtype E, in which the sequence is complementary to the sequence from 7313 to 7294, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

10U/11WE

primer 10U: CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20)

primer 11WE: CTCTACAATTAAAATGATGCATTG (Sequence ID No. 30)

Primer 11WE is a primer specific to only subtype E, in which the sequence is complementary to the sequence from 7352 to 73339, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer pairs and their nucleotide sequences for second PCR to detect subtype C

10C/11RC

primer 10C: AAATGGTAGCCTAGCAGAAG (Sequence ID No. 10)

primer 11RC: CTCCTGAGGATGGTGCAAATTT (Sequence ID No. 13)

Primer 10C is a subtype C and F-specific primer in which the sequence is complementary to the sequence from 6997 to 7016, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11RC is a primer specific to only subtype C, in which the sequence is complementary to the sequence from 7313 to 7292, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

10U/11XC

primer 10U: AAATGGTAGCCTAGCAGAAG (Sequence ID No. 10)

primer 11XC: TTGTTTTATTAGGGAAGTGTTC (Sequence ID No. 29)

Primer 11XC is a primer specific to only subtype C, in which the sequence is complementary to the sequence

from 7289 to 7268, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer pairs and their nucleotide sequences for second PCR to detect subtype D

10/11RD

primer 10: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11RD: CTCCTGAGGATGGTTTAAAAAT (Sequence ID No. 14)

Primer 11RD is a primer specific to only subtype D, in which the sequence is complementary to the sequence from 7313 to 7292, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer pairs and their nucleotide sequences for second PCR to detect subtype F

10C/11RF

primer 10C: AAATGGTAGCCTAGCAGAAG (Sequence ID No. 10)

primer 11RF: CTCCTGAGGATGAGTTAAATTT (Sequence ID No. 15)

Primer 11RF is a primer specific to only subtype F, in which the sequence is complementary to the sequence from 7313 to 7292, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer pairs and their nucleotide sequences for second PCR to detect subtype G

10G/11SG

primer 10G: GAATGGCAGTTTAGCAGAAG (Sequence ID No. 11)

primer 11SG: TCCTGCAGATGAGTTAAAGG (Sequence ID No. 16)

Primer 10G is a primer specific to only subtype G, in which the sequence is complementary to the sequence from 6997 to 7016, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11SG is a primer specific to only subtype G, in which the sequence is complementary to the sequence from 7312 to 7293, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer pairs and their nucleotide sequences for second PCR to detect subtype H

10H/11SH

primer 10H: GTCAAATGGCAGTTTAGCAG (Sequence ID No. 12)

primer 11SH: TCCTGAGGATGGTTTAAAGG (Sequence ID No. 17)

Primer 10H is a primer specific to only subtype H, in which the sequence is complementary to the sequence from 6994 to 7013, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11SH is a primer specific to only subtype H, in which the sequence is complementary to the sequence

from 7312 to 7293, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Second PCR may alternatively be carried out using a mixture of primers capable of giving amplified products for several subtypes in order to permit the amplification of any subtype. Examples of primers for that purpose include the following primer mixtures.

primer 10U: AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4)

primer 11LB: AATTTCTGGGTCCCCTCCTG (Sequence ID No. 18)

primer 11LAE: AATTTCTAGATCCCCTCCTG (Sequence ID No. 25)

primer 11LC: AATTTCTAGGTCCCCTCCTG (Sequence ID No. 26)

Primer 11LB is a subtype B, D, F, G, and I-specific primer in which the sequence is complementary to the sequence from 7327 to 7308, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11LAE is a subtype A, E, F, G, I, and J-specific primer in which the sequence is complementary to the sequence from 7327 to 7308, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11LC is a subtype C, F, G, H, I, and J-specific primer in which the sequence is complementary to

the sequence from 7327 to 7308, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

The following primers can also be used.

Primer 10KC: CTCAACTACTGTTAAATGGTAG (Sequence ID No. 21)

Primer 10KC is a primer specific to only subtype C, in which the sequence is complementary to the sequence from 6984 to 7005, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 10UF: CTGTTAAATGGCAGCCTAGC (Sequence ID No. 22)

Primer 10UF is a subtype A, E, F, H, and I-specific primer in which the sequence is complementary to the sequence from 6992 to 7011, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 10UG: CTGTTAAATGGCAGTTTAGC (Sequence ID No. 23)

Primer 10UG is a subtype A, E, G, I, and J-specific primer in which the sequence is complementary to the sequence from 6992 to 7011, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 10UC: CTGTTAAATGGTAGTCTAGC (Sequence ID No. 24)

Primer 10UC is a subtype C and E-specific primer in which the sequence is complementary to the sequence from

6992 to 7011, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11LE: AATTTCTAGATCTCCTCCTG)Sequence ID No. 19)

Primer 11LE is a subtype E, F, G, H, and J-specific primer in which the sequence is complementary to the sequence from 7327 to 7308, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11LC: AATTTCTAGGTCCCCTCCTG (Sequence ID No. 26)

Primer 11LC is a subtype C, F, G, H, I, and J-specific primer in which the sequence is complementary to the sequence from 7327 to 7308, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11TC: TTCTCCTCTACAATTAAAGC (Sequence ID No. 31)

Primer 11TC is a primer specific to only subtype C, in which the sequence is complementary to the sequence from 7357 to 7338, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11RC1: TTATTGTTTTATTAGGGAAGTG (Sequence ID No. 32)

Primer 11RC1 is a primer specific to only subtype C, in which the sequence is complementary to the sequence from 7292 to 7271, counting from the 5' terminal (left

terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11SE: TGCATTGTAATTTCTAGATCTC (Sequence ID No. 33)

Primer 11SE is a primer specific to only subtype E, in which the sequence is complementary to the sequence from 7333 to 7314, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

Primer 11BE: TGATGCATTGTAATTTCTAG (Sequence ID No. 34)

Primer 11BE is a primer specific to only subtype E, in which the sequence is complementary to the sequence from 7338 to 7319, counting from the 5' terminal (left terminal), of the complete base sequence for HIV-1 (NL4-3 strain).

The PCR procedures and reaction conditions may be in accordance with those in Bruisten S. et al., *AIDS Res Hum Retroviruses* 1993, 9:259-265, but the hot start method is preferred. In hot start PCR, the PCR reaction solution is kept on a hot plate for start up at an elevated temperature (usually 90°C or more).

However, it sometimes happens that no subtype is detected in attempts to determine the HIV-1 subtype in such a method. Possible causes may be that the HIV-1 DNA concentration is below the detection threshold, or the presence of numerous variants at the primer binding site.

To deal with the former possibility, the above method can be implemented after the extraction of the RNA from plasma, since the concentration of HIV-1 is generally higher in plasma than in cells, and its subsequent conversion to DNA using reverse transcriptase.

To deal with the latter possibility, the determination of the subtype by this method is held off, another genetic region of HIV-1 is amplified by PCR to determine the nucleotide sequence, and the subtype is determined by a conventional method (Note: HIV-1 infection is generally diagnosed by detecting antibodies. This invention is not a method for diagnosing HIV-1 infection.).

The PCR reaction products are separated by agarose gel electrophoresis and detected by ethidium bromide staining. Although distinct bands can be observed with the use of primers consistent with the subtype of the HIV-1 in sample DNA, the bands are indistinct or not observed at all when the primers are not. The HIV-1 subtype is determined in this way.

Determining the HIV-1 subtype in the present invention may include the steps of amplifying nucleic acid using as a target sequence a portion of a nucleotide sequence of the HIV-1 genome, where the nucleotide sequence is highly conserved among all subtypes, and determining the presence of absence of HIV-1 depending on

whether or not the nucleic acid has been amplified. The step for ascertaining the presence or absence of HIV-1 may comprise amplifying the nucleic acid with a primer mixture for first PCR (such as 9AE/9B/12A/12B) using as a target sequence a portion of a nucleotide sequence of the HIV-1 genome, the nucleotide sequence being highly conserved among all subtypes, then carrying out a second amplifying reaction with a primer mixture for second PCR (such as 10U/11LB/11LAE/11LC) using as a target sequence a nucleotide sequence in the above target sequence, and then ascertaining the presence or absence of HIV-1 depending on whether or not the nucleic acid has been amplified.

The present invention also encompasses a kit for determining HIV-1 subtypes, comprising primer pairs in which a target sequence is a portion of a nucleotide sequence of the env gene of HIV-1, where at least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the subtype. Examples of primer pairs include primer pairs (inner primers) for second PCR such as those above, and combinations of primer pairs for first PCR (outer primers) and primer pairs for second PCR. The kit of the present invention may also include dNTP mixtures, reaction buffers, DNA polymerase, universal subtype primer pairs for first PCR, and universal subtype primer pairs for second PCR. To minimize the effects

caused by inconsistencies between the primer and analyte HIV-1 DNA base pairs, the magnesium ion concentration of the reaction buffer should be increased from the usual concentration of 1.5 mM to 4 mM.

The components constituting the diagnostic kit may be packaged individually, assembled, or bundled in containers such as vials and tubes, and the containers may be bundled and housed in support means divided for housing such components.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention is illustrated in detail in the following examples, but the scope of the present invention is not in any way limited by these examples.

Example 1

Subjects and Methodology

1) Subtype-specific reference specimens to study method for determining subtype

Reference specimens were prepared after the extraction of DNA from the blood of 3, 8, and 3 HIV-1-infected patients; the subtypesof HIV-1 were determined to be A, B, and E, respectively, by env gene sequencing and phylogenetic analysis.

2) Subjects for determining subtype

The HIV-1 subtype was determined in 8 subjects with HIV who either visited or were hospitalized in Tokyo

hospitals.

3) Preparation of DNA from blood of HIV patients

10 mL peripheral blood was drawn from the above HIV patients. Sodium citrate was used as an anticoagulant. Monocytes were separated from the peripheral blood by Ficoll-Paque (Pharmacia) gradient centrifugation, and DNA was then prepared using a QIAamp Blood Kit (QIAGEN). The DNA was dissolved in pure water or buffer containing 1 mM EDTA, and was stored at -20°C until use. 0.5 μg DNA was used in PCR.

4) Detection of subtypes A, B, and E by PCR

Figure 3 gives the nucleotide sequences of the primers used in PCR.

For subtype A-specific detection, nested PCR was carried out using 9AE and 12A as the primers for first PCR, and 10 and 11QA as the primers for second PCR. For subtype B-specific detection, nested PCR was carried out using 9B and 12B as the primers for first PCR, and 10 and 11BB as the primers for second PCR. For subtype E-specific detection, nested PCR was carried out using 9AE and 12E as the primers for first PCR, and 10 and 11QE as the primers for second PCR (Figure 3).

PCR was carried out for 30 cycles, where one cycle was 15 seconds at 94°C, 30 seconds at 56°C, and 1 minute at 72°C, with 100 μL reaction solution (10 mM Tris-HCl pH

8.3, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM dNTP, 1.0 μM primer, 2.5 units Taq polymerase) using 0.5 μg sample DNA prepared from HIV patients. Second PCR was carried out for 25 cycles under the same conditions using 2 μL reaction solution from the first PCR. 30 seconds at 60°C was used instead of 30 seconds at 56°C, however.

PCR products (subtypes A and E: 317 bp; subtype B: 342 bp) were detected by isolation through electrophoresis with 2% agarose gel, and by subsequent ethidium bromide staining.

Results

1) Study of subtype determination by PCR of subtype-specific reference samples

The following results were obtained for specimens whose subtype had been determined by virus genome sequencing. Only subtype A specimens were positive, while subtype B and E specimens were all negative, in PCR using primers for the detection of subtype A. Only subtype B specimens were positive, while subtype A and E specimens were all negative, in PCR using primers for the detection of subtype B. Only subtype E specimens were positive, while subtype A and B specimens were all negative, in PCR using primers for the detection of subtype E (Figure 4).

2) Determination of HIV patient subtype by PCR

Table 1 gives the results obtained in the

determination of HIV-1 subtypes in 8 HIV patients who either visited or were hospitalized in Tokyo hospitals.

Table 1: results obtained in determination of subtypes in 8 specimens of unknown subtype

Case	primer pair for subtype A	primer pair for subtype B	primer pair for subtype E
P18	—	+	—
P19	—	+	—
P20	—	+	—
P21	—	—	+
P22	—	?	—
P23	—	—	+
P24	—	+	—
P25	—	+	—

In the table above, + denotes detection of HIV-1 specific DNA bands, - denotes non-detection thereof. The symbol ? for case P22 denotes detection of shorter bands than prediction.

Based on these results, Cases P18, P19, P20, P24, and P25 were diagnosed as being infected with subtype B, and Cases P21 and P23 were diagnosed as being infected with subtype E. Although a DNA band was detected only with the use of a primer pair for subtype B in Case P22, it was shorter than expected, so determination was postponed. To verify that the above results were correct, the amplified DNA was sequenced and phylogenetically analyzed, giving results that were consistent with those in Table 1. Case P22 turned out to be subtype B.

The results in 1) and 2) demonstrate that this

method was able to correctly diagnose the subtype in 21 out of 22 cases. The determination was postponed in the remaining one case. That is, the present method has been shown to be a simple and reliable method for determining subtypes.

The method of the present invention allows HIV-1 subtypes to be determined at a cost of about ¥2,000 per specimen. The time needed to determine the subtype in treating all 8 specimens at once was 2 hour for the isolation of the DNA, 6 hours for PCR, and about 1 hour for electrophoresis.

Example 2

Subjects and Methodology

1) Subtype-specific reference specimens for detection in subtype determination method

Reference specimens were prepared from DNA extracted from the blood of 11 subjects, which included 2 patients with HIV-1 determined to be subtype C by env gene sequencing and phylogenetic analysis, in addition to the 3 subtype A HIV-1 subjects, 3 subtype B HIV-1 subjects, and 3 subtype E HIV-1 subjects used in Example 2.

2) Subjects for determining subtype

The HIV-1 subtype was determined in 32 subjects with HIV who either visited or were hospitalized in Tokyo hospitals.

3) Preparation of DNA from blood of HIV patients

10 mL peripheral blood was drawn from the above HIV patients. Sodium citrate was used as an anticoagulant. Monocytes were separated from the peripheral blood by Ficoll-Paque (Pharmacia) gradient centrifugation, and DNA was then prepared using a QIAamp Blood Kit (QIAGEN). The DNA was dissolved in pure water or buffer containing 1 mM EDTA, and was stored at -20°C until immediately before use. 0.5 μg DNA was used in PCR.

4) Detection of subtypes A, B, C, and E by PCR

Figure 5 gives the nucleotide sequences of the primers used in PCR.

A mixture of 9AE, 9B, 12A, and 12B was used for the primers in first PCR. Nested PCR was carried out using the following primers for second PCR: 10U and 11QA1 for subtype A-specific detection, 10U and 11VB for subtype B-specific detection, 10U and 11XC for subtype C-specific detection, and 10U and 11WE for subtype E-specific detection. Nested PCR was carried out using a mixture of 10U, 11LB, 11LAE, and 11LC for amplification of HIV-1 DNA irrespective of subtype (Figure 5).

PCR was carried out for 30 cycles, where one cycle was 15 seconds at 94°C, 30 seconds at 56°C, and 1 minute at 72°C, with 100 μL reaction solution (10 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM dNTP, 1.0 μM primer,

2.5 units Taq polymerase) using 0.5 μg sample DNA prepared from HIV patients. Second PCR was carried out for 25 cycles under the same conditions using 2 μL reaction solution from the first PCR. 30 seconds at 60°C was used instead of 30 seconds at 56°C, however.

PCR products (subtype A: 322 bp; subtype B: 358 bp; subtype C: 298 bp; subtype E: 361)) were detected by isolation through electrophoresis with 2% agarose gel, and by subsequent ethidium bromide staining.

Results

1) Study of subtype by PCR of subtype-specific reference samples

The following results were obtained for specimens whose subtype had been determined by virus genome sequencing. Only subtype A specimens were positive, while subtype B, C, and E specimens were all negative, in PCR using primers for the detection of subtype A. Only subtype B specimens were positive, while subtype A, C, and E specimens were all negative, in PCR using primers for the detection of subtype B. Only subtype C specimens were positive, while subtype A, B, and E specimens were all negative, in PCR using primers for the detection of subtype C. Only subtype E specimens were positive, while subtype A, B, and C specimens were all negative, in PCR using primers for the detection of subtype E (Figure 6).

All specimens were positive in PCR using primers for amplifying HIV-1 DNA irrespective of subtype (Figure 6).

2) Determination of subtype of HIV patients by PCR

The HIV-1 subtype was determined in 32 HIV patients who either visited or were hospitalized in Tokyo hospitals. Together with the 11 subtype-specific reference samples, there were 3 subtype A cases, 30 subtype B cases, 2 subtype C cases, and 8 subtype E cases.

To verify that the above results were correct, the amplified DNA of 21 out of the 43 HIV patients whose subtype was determined by PCR was sequenced and phylogenetically analyzed, giving the results shown in Figure 7. There was complete agreement between the subtypes determined by phylogenetic analysis and the subtypes determined by PCR for these cases of HIV-1.

The method employed in Example 2 differs significantly from that of Example 1 in that universal primers were used in first PCR, and subtype-specific primers were used in second PCR. As a result, the number of PCR reactions could be reduced to 5/8. In view of the fact that the determination of the subtype had to be postponed in 1 case in Example 1, the method of Example 2 was able to provide more accurate diagnosis and was also simpler.

3) Effect of subtype on testing for drug resistance

by genotype

Drug resistance by genotype was analyzed based on data for subtype B. To investigate whether or not the drug resistance of HIV-1 subtypes other than subtype B could be determined using data for subtype B, the amino acid sequences for HIV-1 protease before and after HAART treatment were determined in 4 patients infected with HIV-1 other than subtype B who were receiving HAART treatment. After HAART treatment in case C3, which was subtype E, amino acid No. 10 had mutated from L (leucine) to F (phenylalanine), and amino acid No. 20 had mutated from K (lysine) to T (threonine). This was recognized as an amino acid mutation indicative of drug resistance in subtype B. However, in all four subjects, amino acid No. 36 was I (isoleucine) from before HAART treatment. In the data for subtype B, HIV-1 with I as the No. 36 amino acid was interpreted as indicative of drug resistance. However, it is difficult to conclude that HIV-1 would have acquired drug resistance before administration of the drug. It would be more logical to view this mutation as irrelevant to drug resistance in HIV-1 subtypes other than subtype B. It may thus be concluded that it is important to diagnose the subtype in advance in order to properly assess drug resistance by genotype.

4) Relationship between subtype and sexual behavior

Figure 9 summarizes the relation between subtype and sexual behavior in 22 patients with HIV who had been interviewed about their sexual preferences. Heterosexuals are those attracted to the opposite sex, while MSM are male homosexuals. About the same number of heterosexuals were subtype B and E, whereas male homosexuals were far more likely to be subtype B. This would seem to indicate that Southeast Asian HIV has spread among heterosexuals but has not spread very much among male homosexuals.

Example 3

Subjects and Methodology

1) Western blot-negative and PA-positive serum specimens

The specimens were 15 serum samples whose blood tests at Tokyo hospitals showed to be HIV-1 negative by Western blotting and HIV-1 positive by PA.

2) Preparation of DNA from plasma RNA

RNA was prepared using an RNAeasy Kit (QIAGEN) from 200 μL of the aforementioned serum specimen. The RNA was dissolved in pure water and stored at -20°C until immediately before use.

3) Detection of HIV-1 by PCR

RNA corresponding to 20 μL portions of serum was used as the material, and cDNA was synthesized by 30 minutes of reaction at 42°C using a mixture of primers 12A

and 12B with 20 μL reaction solution (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM dNTP, 5 μM primer, and 100 unites reverse transcriptase). The cDNA was used as material in nested PCR capable of amplifying the DNA of HIV-1 irrespective of subtype. A mixture of 9AE, 9B, 12A, and 12B was used for the primers in first PCR. A mixture of 10U, 11LB, 11LAE, and 11LC was used for the primers in second PCR.

First PCR was carried out for 30 cycles, where one cycle was 15 seconds at 94°C, 30 seconds at 56°C, and 1 minute at 72°C, with 100 μL reaction solution (10 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM dNTP, 1.0 μM primer, 2.5 units Taq polymerase). Second PCR was carried out for 25 cycles under the same conditions using 2 μL reaction solution from the first PCR. 30 seconds at 60°C was used instead of 30 seconds at 56°C, however.

PCR products (subtype A: 322 bp; subtype B: 358 bp; subtype C: 298 bp; subtype E: 361) were detected by isolation through electrophoresis with 2% agarose gel, and by subsequent ethidium bromide staining.

Results

No HIV-1 was detected by PCR in any of the 15 serum specimens which were HIV-1 negative by Western blotting and HIV-1 positive by PA (Figure 10). It was proven (Figure 6) that the PCR run here was designed to enable

detection of all HIV-1 subtypes. It is highly possible that the inconsistency between the results by Western blotting and PA for the serum specimens tested here is not a problem of subtype, but that the PA method resulted in false positive results.

PCR enabling the detection of HIV-1 irrespective of subtype may therefore be more effective for reliable diagnosis of HIV-1 infection.

The present invention provides a simple method for determining HIV-1 subtypes.

The invention also provides an effective means for determining HIV-1 subtypes.

Sequence ID No. 1 gives the nucleotide sequence for primer 11QA.

Sequence ID No. 2 gives the nucleotide sequence for primer 11BB.

Sequence ID No. 3 gives the nucleotide sequence for primer 11QE.

Sequence ID No. 4 gives the nucleotide sequence for primer 10.

Sequence ID No. 5 gives the nucleotide sequence for primer 12A.

Sequence ID No. 6 gives the nucleotide sequence for primer 12B.

Sequence ID No. 7 gives the nucleotide sequence for

primer 12E.

Sequence ID No. 8 gives the nucleotide sequence for primer 9AE.

Sequence ID No. 9 gives the nucleotide sequence for primer 9B.

Sequence ID No. 10 gives the nucleotide sequence for primer 10C.

Sequence ID No. 11 gives the nucleotide sequence for primer 10G.

Sequence ID No. 12 gives the nucleotide sequence for primer 10H.

Sequence ID No. 13 gives the nucleotide sequence for primer 11RC.

Sequence ID No. 14 gives the nucleotide sequence for primer 11RD.

Sequence ID No. 15 gives the nucleotide sequence for primer 11RF.

Sequence ID No. 16 gives the nucleotide sequence for primer 11SG.

Sequence ID No. 17 gives the nucleotide sequence for primer 11SH.

Sequence ID No. 18 gives the nucleotide sequence for primer 11LB.

Sequence ID No. 19 gives the nucleotide sequence for primer 11LE.

Sequence ID No. 20 gives the nucleotide sequence for primer 10U.

Sequence ID No. 21 gives the nucleotide sequence for primer 10KC.

Sequence ID No. 22 gives the nucleotide sequence for primer 10UF.

Sequence ID No. 23 gives the nucleotide sequence for primer 10UG.

Sequence ID No. 24 gives the nucleotide sequence for primer 10UC.

Sequence ID No. 25 gives the nucleotide sequence for primer 11LAE.

Sequence ID No. 26 gives the nucleotide sequence for primer 11LC.

Sequence ID No. 27 gives the nucleotide sequence for primer 11QA1.

Sequence ID No. 28 gives the nucleotide sequence for primer 11VB.

Sequence ID No. 29 gives the nucleotide sequence for primer 11XC.

Sequence ID No. 30 gives the nucleotide sequence for primer 11WE.

Sequence ID No. 31 gives the nucleotide sequence for primer 11TC.

Sequence ID No. 32 gives the nucleotide sequence for

primer 11RC1.

Sequence ID No. 33 gives the nucleotide sequence for primer 11SE.

Sequence ID No. 34 gives the nucleotide sequence for primer 11BE.

CLAIMS

1. A method for determining HIV-1 subtypes, characterized by comprising the steps of amplifying nucleic acid using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1, where at least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the HIV-1 subtype, and detecting the subtype depending on whether or not the nucleic acid has been amplified.

2. The method according to Claim 1, wherein the target sequence is 100 to 2500 nucleotides long.

3. The method according to Claim 1, wherein the sequence from the 1st through 30th bases from the 3' terminal and/or 5' terminal of the target sequence is different depending on the subtype.

4. The method according to Claim 3, wherein the 3' terminal of the target sequence is in the C3 region of the env gene of HIV-1.

5. The method according to Claim 4, wherein the 5' terminal of the target sequence is in the C2 region of the env gene of HIV-1.

6. The method according to Claim 1, wherein different amplification reactions are carried out using different pairs of primers, and different subtypes are detected.

7. The method according to Claim 6, wherein at least two different subtypes are detected by carrying out amplification at least twice with different pairs of primers using primer pairs consisting of a primer (primer 1) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 1) that differs depending on subtype in the C3 region of the env gene of HIV-1, and a primer (primer 2) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 2) of the C2 region of the env gene of HIV-1.

8. The method according to Claim 1, wherein a first amplification reaction is carried out with a first pair of primers using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1, a second amplification reaction is then carried out with a second pair of primers using as a target sequence a portion of said nucleotide sequence, where at least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the HIV-1 subtype, and the subtype is detected depending on whether or not the nucleic acid has been amplified by the second amplification reaction.

9. The method according to Claim 8, wherein the second pair of primers consists of a primer (primer 1) that includes a sequence complementary to a portion of the

nucleotide sequence (nucleotide sequence 1) that differs depending on subtype in the C3 region of the env gene of HIV-1, and a primer (primer 2) that includes a sequence complementary to a portion of the nucleotide sequence (nucleotide sequence 2) of the C2 region of the env gene of HIV-1; and the first pair of primers consists of a primer (primer 3) that includes a sequence complementary to a portion of a nucleotide sequence (nucleotide sequence 3) of a region downstream of the 3' terminal of nucleotide sequence 1 of the env gene of HIV-1, and a primer (primer 4) that includes a sequence complementary to a portion of a nucleotide sequence (nucleotide sequence 4) of a region upstream of the 5' terminal of nucleotide sequence 2 of the env gene of HIV-1.

10. The method according to Claim 8, wherein at least two subtypes are distinguished by repeating at least once, with different pairs of second primers, a series of operations comprising: a first amplification reaction that is carried out with the first pair of primers using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1; a second amplification reaction that is then carried out with the second pair of primers using as a target sequence a nucleotide sequence within said target sequence; and the detection of subtypes depending on whether or not the nucleic acid has been amplified by the

second amplification reaction.

11. The method according to Claim 10, wherein subtypes A, B, C, and E are distinguished by:

(a) detecting subtype A using as the first primer pair a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6), and a mixture of primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair primer 11QA1 containing nucleotide sequence CTCCTGAGGAGTTAGCAAAG (Sequence ID No. 27) and primer 10U containing nucleotide sequence CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20);

(b) detecting subtype B using as the first primer pair a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6), and a mixture of primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair

primer 11VB containing nucleotide sequence CACAATTAAAACTGTGCATTAC (Sequence ID No. 28) and primer 10U containing nucleotide sequence CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20);

(c) detecting subtype C using as the first primer pair a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6), and a mixture of primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair primer 11XC containing nucleotide sequence TTGTTTTATTAGGGAAGTGTTC (Sequence ID No. 29) and primer 10UC containing nucleotide sequence CTGTTAAATGGTAGTCTAGC (Sequence ID No. 24); and

(d) detecting subtype E using as the first primer pair a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6), and a mixture of primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG

(Sequence ID No. 9), and using as the second primer pair primer 11WE containing nucleotide sequence CTCTACAATTAAAATGATGCATTG (Sequence ID No. 30) and primer 10U containing nucleotide sequence CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20).

12. The method according to Claim 8, wherein at least two subtypes are distinguished by repeating at least once, with different pairs of first and second primers, a series of operations comprising: a first amplification reaction that is carried out with a first pair of primers using as a target sequence a portion of a nucleotide sequence of the env gene of HIV-1; a second amplification reaction that is then carried out with a second pair of primers using as a target sequence a nucleotide sequence within said target sequence; and the detection of subtypes depending on whether or not the nucleic acid has been amplified by the second amplification reaction.

13. The method according to Claim 12, wherein subtypes A, B, and E are distinguished by:

(a) detecting subtype A using as the first primer pair primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5) and primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8), and using as the second primer pair primer 11QA containing nucleotide sequence

CTCCTGAGGGGTTAGCAAAG (Sequence ID No. 1) and primer 10 containing nucleotide sequence AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4);

(b) detecting subtype B using as the first primer pair primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6) and primer 9B containing nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and using as the second primer pair primer 11BB containing nucleotide sequence CTGTGCATTACAATTTCTGG (Sequence ID No. 2) and primer 10 containing nucleotide sequence AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4); and

(c) detecting subtype E using as the first primer pair primer 12E containing nucleotide sequence GCAATAGAAAAATTCCCCTC (Sequence ID No. 7) and primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG (Sequence ID No. 8), and using as the second primer pair primer 11QE containing nucleotide sequence CTCCTGAGGGTGGTTGAAAG (Sequence ID No. 3) and primer 10 containing nucleotide sequence AAATGGCAGTCTAGCAGAAG (Sequence ID No. 4).

14. The method according to Claim 1, further comprising the steps of amplifying nucleic acid using as a target sequence a portion of a nucleotide sequence of the HIV-1 genome, the nucleotide sequence being highly

conserved among all subtypes, and ascertaining the presence or absence of HIV-1 depending on whether or not the nucleic acid has been amplified.

15. The method according to Claim 14, wherein the step for ascertaining the presence or absence of HIV-1 comprises amplifying the nucleic acid with a first primer pair using as a target sequence a portion of a nucleotide sequence of the HIV-1 genome, the nucleotide sequence being highly conserved among all subtypes, then carrying out a second amplifying reaction with a second primer pair using as a target sequence a nucleotide sequence in said target sequence, and ascertaining the presence or absence of HIV-1 depending on whether or not the nucleic acid has been amplified.

16. The method according to Claim 15, wherein the primers that are used comprise a mixture of a plurality of upstream primers with differing nucleotide sequences and a plurality of downstream primers with differing nucleotide sequences.

17. The method according to Claim 16, wherein the first primers comprise a mixture of primer 12A containing nucleotide sequence GCAATAGAAAAATTCTCCTC (Sequence ID No. 5), primer 12B containing nucleotide sequence ACAGTAGAAAAATTCCCCTC (Sequence ID No. 6), primer 9AE containing nucleotide sequence CACAGTACAATGCACACATG

(Sequence ID No. 8), and primer 9B nucleotide sequence CACAGTACAATGTACACATG (Sequence ID No. 9), and the second primer pair comprises primer 11LB containing nucleotide sequence AATTTCTGGGTCCCCTCCTG (Sequence ID No. 18), primer 11LAE containing nucleotide sequence AATTTCTAGATCCCCTCCTG (Sequence ID No. 25), primer 11LC containing nucleotide sequence AATTTCTAGGTCCCCTCCTG (Sequence ID No. 26), and primer 10U containing nucleotide sequence CTGTTAAATGGCAGTCTAGC (Sequence ID No. 20).

18. A kit for determining HIV-1 subtypes, comprising primer pairs in which a target sequence is a portion of a nucleotide sequence of the env gene of HIV-1, where at least one of the 5' terminal and 3' terminal nucleotide sequences is different depending on the subtype.

Fig. 1

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SUBTYPE A   TGTAataccTCAgccatTAcAcAgGCtTGtCCaAAggTatCCTTTGAgCCaATTCCCATA
SUBTYPE B   -----c-------t------------c---------------------------------
SUBTYPE C   -----------------A-----a--C-----------c--t-----c--------T---
SUBTYPE D   --------------------------g------------a--------------------
SUBTYPE E   --------T----T-----aG---------------a----------T--------t---
SUBTYPE F   ------------A----------------------------T-GG--T------------
SUBTYPE G   ------gt----A------A------------------ga?T-----c------------
SUBTYPE H   ------GT-----------A------------------GAGT-----T------------

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SUBTYPE B   --------------g--------------------------t------a-----------
SUBTYPE C   -----------t----------a------------------ta-----aca--------g
SUBTYPE D   --------------------a-----a--------a------------A----------g
SUBTYPE E   ----------a-t---------a-------t----------T------a-t--------g
SUBTYPE F   ------------T---------A------------------T-----aA----------G
SUBTYPE G   ------------T-----------------t---------gg------a-?---------
SUBTYPE H   ------------T-------G---------A---------GG------A----------G

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SUBTYPE B   -----a-----t-ca--------------------t-----------t-g----------
SUBTYPE C   -----a------c-t--------------------t-----------t--------g---
SUBTYPE D   -----?--------a--------------------t--------g--t-g---------g
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SUBTYPE F   --g--------------------------------T-----------T--A-----g---
SUBTYPE G   -----A-----T--a--------T-----------T-----------T--------g---
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SUBTYPE B   ---------------------------------------...------g-a---------
SUBTYPE C   -----------a-----------t--c------------...---a----a---------
SUBTYPE D   ------------?----g---------------------...---a----A---------
SUBTYPE E   ---------t-----------------------------...---A----A--c------
SUBTYPE F   ---------T--T-------------C------------...--ta----A--c------
SUBTYPE G   ---------t-a---c-g---------t-----------...--aA----a---------
SUBTYPE H   ---------T-A---C-GTCAAATG-CAGTTT--C---------?a----a---------

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SUBTYPE C   ------c-g----------t------a-----------t---aAtg-at-----ga----
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SUBTYPE E   ------C----------------------------G--C---AAT--At-----Ga---C
SUBTYPE F   c--------t--g-t-----A-----------------?---AATg-At-----ca----
SUBTYPE G   -----c?-----g-----------gt---------g------AAt--a?--a--ga----
SUBTYPE H   -----c------g-----a-----gt----------------AAt--a?-----g-----

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SUBTYPE B   ------
SUBTYPE C   gtg---
SUBTYPE D   ------
SUBTYPE E   ------
SUBTYPE F   ------
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SUBTYPE H   --?---
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Fig. 2

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SUBTYPE C   -----cA-T----a-ga?a--------?-----------a-----ag--a-a-----gc-
SUBTYPE D   ------a-T----a-ga?a--------------------c---------a-a-----g--
SUBTYPE E   ---G-gA-T-A-g--A--a--------g--g-----a--c-----a--ga-a------a-
SUBTYPE F   -----c--t---g--a--C----------?--?---g--a-----a?ggc-a-g----ag
SUBTYPE G   --------t------a?-a-t-----?g-g-tG-----ga-t--?a??gc-?--C---a-
SUBTYPE H   --------T------g?-a-?------g-g-tg---?-?a------?-?c-------?a-

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SUBTYPE B   g--??????c-a---...g-g-----t------...---------g-----aa-c-a???
SUBTYPE C   g--......c----ccct--------T------.........----aa-----acca...
SUBTYPE D   g-c?.....cTtc--......------------......aca-----t---aaacCa...
SUBTYPE E   g-g......C--------...a-t--T--G---.........---------caaCCA???
SUBTYPE F   tct......c-t--c---...-----tgc----.....--------aa---aactcA...
SUBTYPE G   g--......at--------------------?c???----------c----aaCtCA...
SUBTYPE H   ---......?--------...a----t----??......-------c----aaacca???

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SUBTYPE B   t------------cCc-------gt--tg--c------------------g---------
SUBTYPE C   t------------cc--------------------c---------a--------------
SUBTYPE D   t------------ccc---------------c------------------g---------
SUBTYPE E   c----------a---C-----------tg---ca-----------A----g---------
SUBTYPE F   t------------CC------------tg----------------a--------------
SUBTYPE G   t-tg---------cC------------------------------a--------------
SUBTYPE H   t------------Cc-------------------?----------a--------------

SUBTYPE A   TAtTGc
SUBTYPE B   --c--t
SUBTYPE C   ------
SUBTYPE D   --C---
SUBTYPE E   ------
SUBTYPE F   --C---
SUBTYPE G   -----t
SUBTYPE H   -----t
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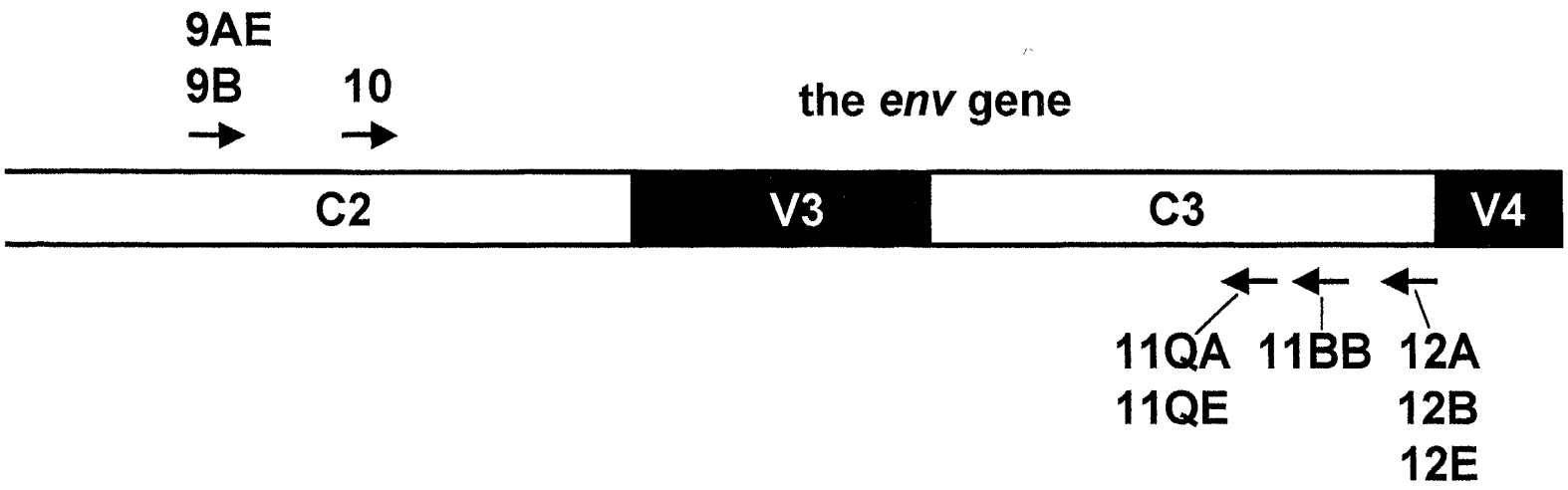


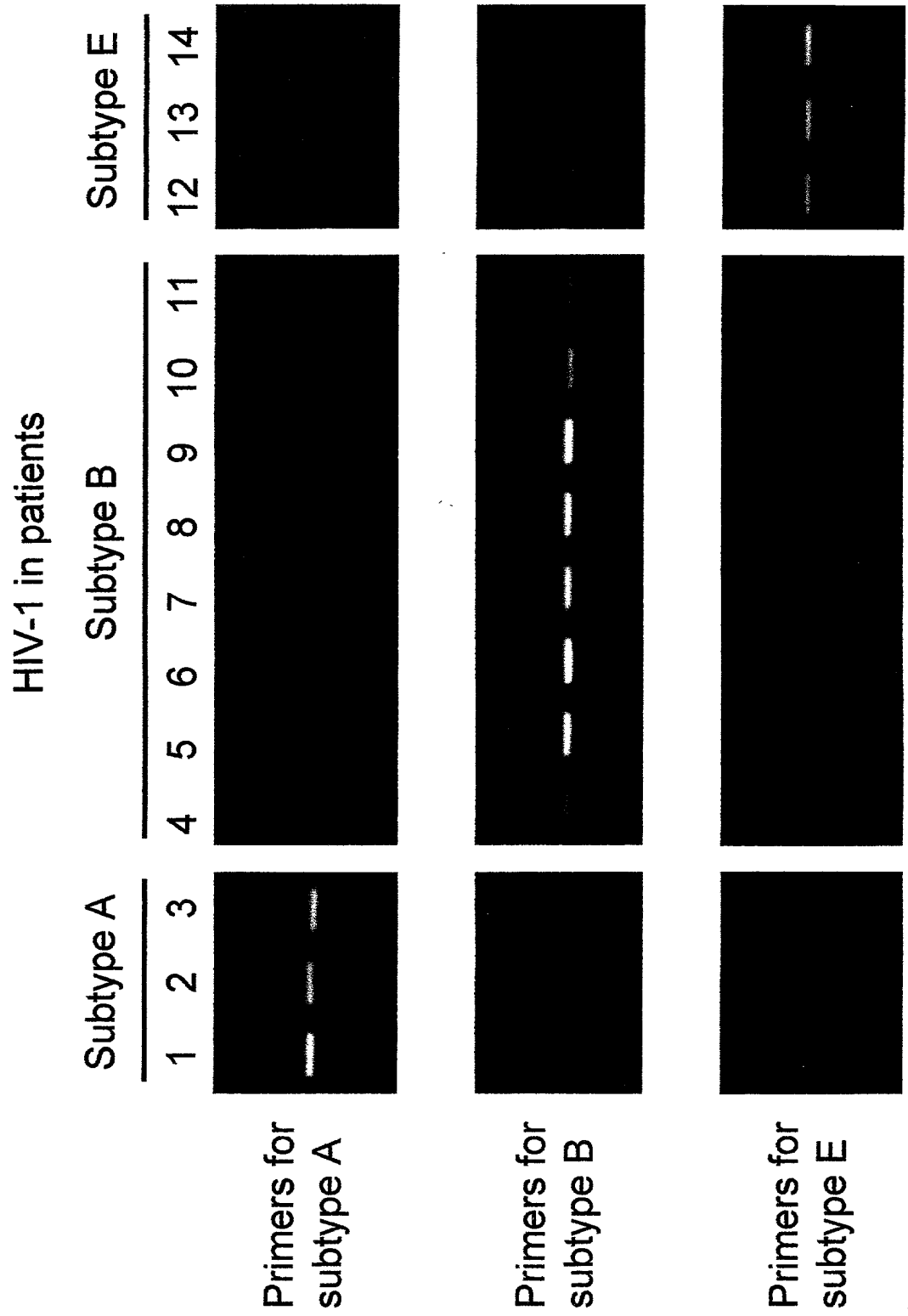
Fig. 3

Primers used for		
Subtype	1st PCR	2nd PCR
A	9AE/12A	10/11QA
B	9B/12B	10/11BB
E	9AE/12E	10/11QE

Primers

9AE:	CACAGTACAATGCACACATG
9B:	CACAGTACAATGTACACATG
10:	AAATGGCAGTCTAGCAGAAG
11QA:	CTCCTGAGGGGTTAGCAAAG
11QE:	CTCCTGAGGGTGGTTGAAAG
11BB:	CTGTGCATTACAATTTCTGG
12A:	GCAATAGAAAAATTCTCCTC
12B:	ACAGTAGAAAAATTCCCCTC
12E:	GCAATAGAAAAATTCCCCTC

Fig. 4



Primers used for Subtype	1st PCR	2nd PCR
A	9M/12M	10U/11QA1
B	9M/12M	10U/11VB
C	9M/12M	10U/11XC
E	9M/12M	10U/11WE
All	9M/12M	10U/11M

Primers

9AE:	CACAGTACAATGCACACATG
9B:	CACAGTACAATGTACACATG
10U:	CTGTTAAATGGCAGTCTAGC
11QA1:	CTCCTGAGGAGTTAGCAAAG
11VB:	CACAATTAAAACTGTGCATTAC
11XC:	TTGTTTTATTAGGGAAGTGTTC
11WE	CTCTACAATTAAAATGATGCATTG
11LAE:	AATTTCTAGATCCCCTCCTG
11LB:	AATTTCTGGGTCCCCTCCTG
11LC:	AATTTCTAGGTCCCCTCCTG
12A:	GCAATAGAAAAATTCTCCTC
12B:	ACAGTAGAAAAATTCCCCTC

10/009897

Fig. 6

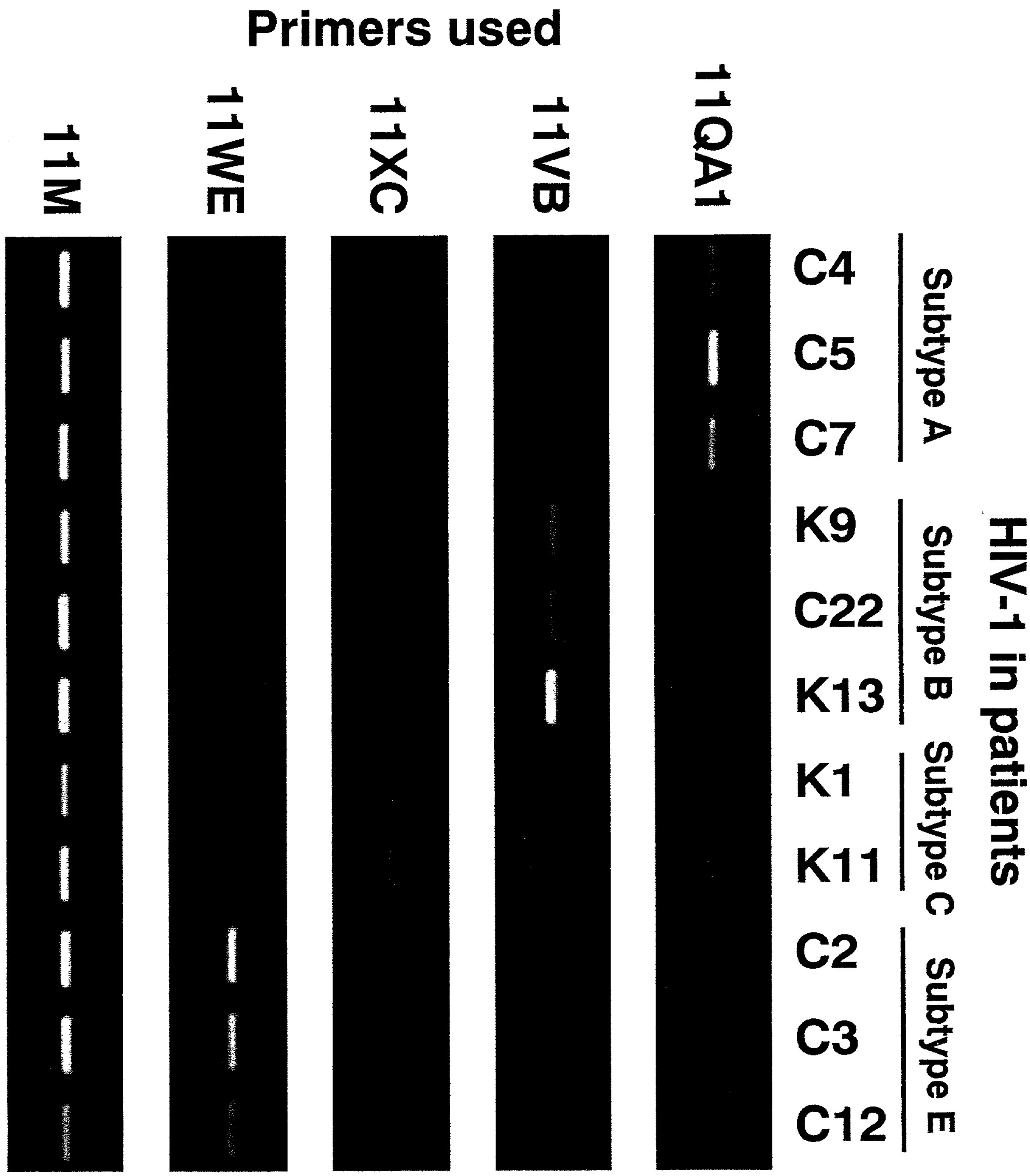
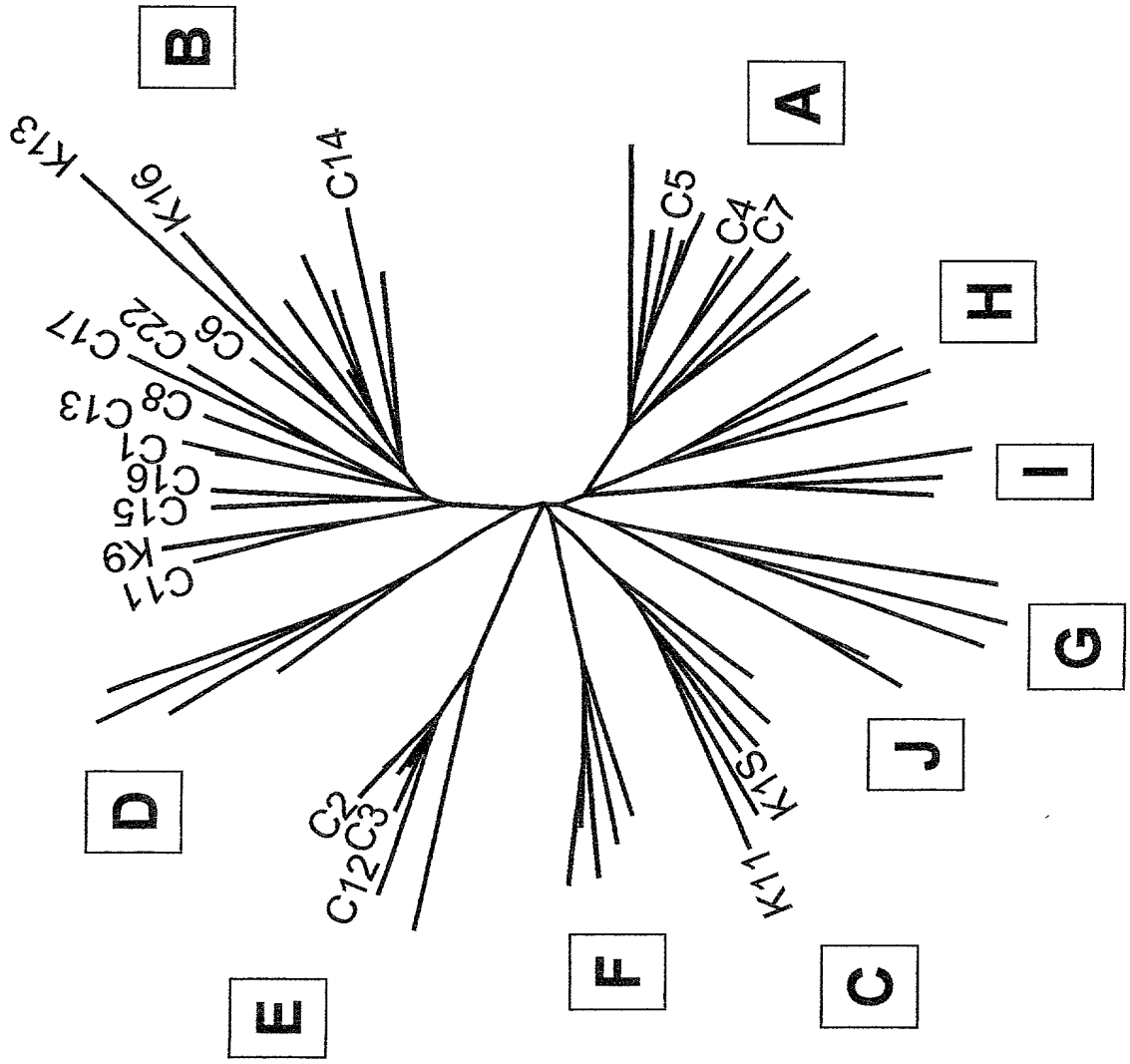


Fig. 7



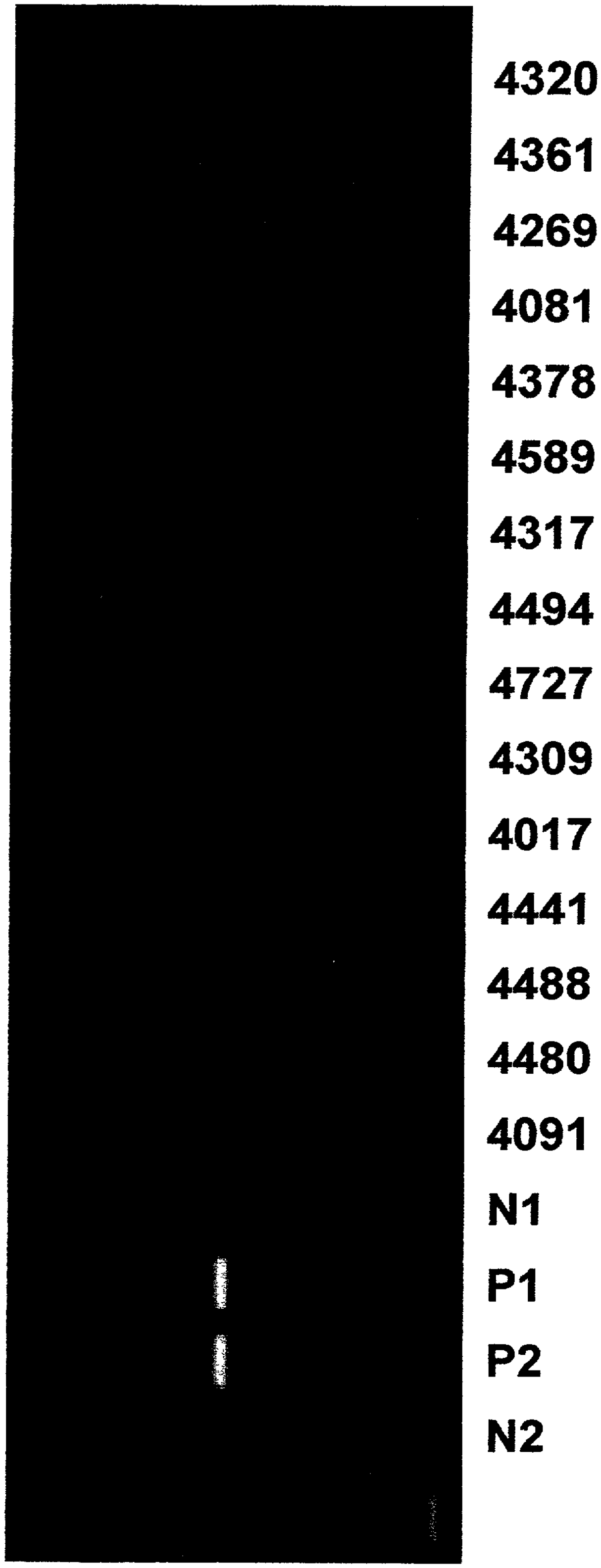
Patient	Subtype	Date	Amino acid at positions relevant to PRI resistance										
			10	20	30	36	46	48	50	63	82	84	90
C3	E	8/11/97	L	K	D	I	M	G	I	L	V	I	L
		6/17/99	F	T	D	I	M	G	I	L	V	I	L
C4	A	6/9/97	L	I	D	I	M	G	I	N	V	I	L
		3/11/98	L	I	D	I	M	G	I	N	V	I	L
C5	A	6/23/97	L	I	D	I	M	G	I	P	V	I	L
		1/11/99	L	I	D	I	M	G	I	P	V	I	L
C7	A	7/29/97	L	I	D	I	M	G	I	N	V	I	L
		9/16/99	L	I	D	I	M	G	I	N	V	I	L

Fig. 8

Fig. 9

	Heterosexual	MSM	Total
Subtype A	2	1	3
Subtype B	6	17	23
Subtype C	2	0	2
Subtype E	6	1	7
Total	16	19	35

Fig. 10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P2
N2

SOLE/JOINT

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name: that I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter claimed and for which a patent is sought in the application entitled:

METHOD FOR DETERMINING HIV-1 SUBTYPE

which application is:

☐ the attached application (for original application)

☐ Application No. _____ filed _____, and amended on _____ (for declaration not accompanying application)

☒ PCT International Application No.PCT/JP00/03896 filed on June 15, 2000

that I have reviewed and understand the contents of the specification of the above-identified application, including the claims, as amended by any amendment referred to above; that I acknowledge my duty to disclose information of which I am aware which is material to the patentability of this application under 37 C.F.R. 1.56, that I hereby claim priority benefits under Title 35, United States Code §119, §172 or §365 of any provisional application or foreign application(s) for patent or inventor's certificate listed below and have also identified on said list any foreign application for patent or inventor's certificate on this invention having a filing date before that of any foreign application on which priority is claimed:

Application Number	Country	Filing Date	Priority Claimed Yes	Priority Claimed No
1999-167736	Japan	15/06/1999	☒	☐
2000-23581	Japan	01/02/2000	☒	☐

I hereby claim the benefit of Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in a listed prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge my duty to disclose any information material to the patentability of this application under 37 C.F.R. 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Filing Date	Status

I hereby appoint John H. Mion, Reg. No. 18,879; Thomas J. Macpeak, Reg. No. 19,292; Robert J. Seas, Jr., Reg. No. 21,092; Darryl Mexic, Reg. No. 23,063; Robert V. Sloan, Reg. No. 22,775; Peter D. Olexy, Reg. No. 24,513; J. Frank Osha, Reg. No. 24,625; Louis Gubinsky, Reg. No. 24,835; Neil B. Siegel, Reg. No. 25,200; David J. Cushing, Reg. No. 28,703; John R. Inge, Reg. No. 26,916; Joseph J. Ruch, Jr., Reg. No. 26,577; Sheldon I. Landsman, Reg. No. 25,430; Richard C. Turner, Reg. No. 29,710; Howard L. Bernstein, Reg. No. 25,665; Alan J. Kasper, Reg. No. 25,426; Kenneth J. Burchfiel, Reg. No. 31,333; Gordon Kit, Reg. No. 30,764; Susan J. Mack, Reg. No. 30,951; Frank L. Bernstein, Reg. No. 31,484; Mark Boland, Reg. No. 32,197; William H. Mandir, Reg. No. 32,156; Brian W. Hannon, Reg. No. 32,778; Abraham J. Rosner, Reg. No. 33,276; Bruce E. Kramer, Reg. No. 33,725; Paul F. Neils, Reg. No. 33,102; Brett S. Sylvester, Reg. No. 32,765; Robert M. Masters, Reg. No. 35,603, George F. Lehnigk, Reg. No. 36,359, John T. Callahan, Reg. No. 32,607; Steven M. Gruskin, Reg. No. 36,818; Peter A. McKenna, Reg. No. 38,551 and Edward F. Kenehan, Reg. No. 28,962, my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and request that all correspondence about the application be addressed to **SUGHRUE, MION, ZINN, MACPEAK & SEAS, PLLC**, 2100 Pennsylvania Avenue, N.W., Washington, D.C. 20037-3213.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Date Sixth Inventor
First Name Middle Initial Last Name

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SEQUENCE LISTING

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